Rapid Methyl Red Technique used in the Confirmation of *Escherichia coli* Cultured from Fish Products

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RAPID METHYL RED TECHNIQUE USED IN THE CONFIRMATION OF *Escherichia coli* CULTURED FROM FISH PRODUCTS

by

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TABLE OF CONTENTS

	Page
ABSTRACT/RESUME	iv
INTRODUCTION	1
Principle and purpose	1
Biochemistry	1
Interpretation	1
COMPARISON TESTING	1
CONCLUSION	2
ACKNOWLEDGMENTS	2
REFERENCES	2

LIST OF APPENDICES

Appendix		Page
1	Standard procedures for bacteriological analyses	3
2	Comparison results of routine MR to rapid MR	4

ABSTRACT

Bell, C. 1983. Rapid methyl red technique used in the confirmation of Escherichia coli cultured from fish products. Can. Manuscr. Rep. Fish. Aquat. Sci. 1709: iv + 4 p.

Tests are necessary to prove that all imported and domestic fish products meet set bacteriological guidelines. Completion of these tests results in a delay to the importer or processor before he can sell the product. The rapid methyl red test was found to provide accurate results within a shortened time frame. This allows the importer or producer to move his products faster and to save money both for himself and the consumer. In the testing facility less bacteriological media is used and less staff time is required to complete the tests.

Key words: fecal coliform analysis; IMViC series; rapid bacteriological identification.

RESUME

Bell, C. 1983. Rapid methyl red technique used in the confirmation of Escherichia coli cultured from fish products. Can. Manuscr. Rep. Fish. Aquat. Sci. 1709: iv + 4 p.

Il faut effectuer des tests pour s'assurer que tous les produits de pêche importés et intérieurs répondent aux normes bactériologiques fixées. Ces tests occasionnent, pour l'importateur ou le transformateur, un retard dans la vente de ses produits. On a découvert que le test rapide au rouge de méthyle pouvait donner des résultats exacts dans un délai plus court, ce qui permet à l'importateur ou au producteur d'écouler ses stocks plus rapidement et ainsi économiser de l'argent, tant pour lui-même que pour le consommateur. En outre, ces tests exigent moins de médium bactériologique et moins d'heures-personnes.

Mots-clés: analyse des bactéries coliformes dans les matières fécales; série IMViC; identification bactériologique rapide.

INTRODUCTION

Imported and domestic fish products are subject to bacteriological inspection to determine if Escherichia coli is present in the product. These results are used to determine the sanitation level of the plant at the time of production and if E. coli levels reach unacceptable levels plant and/or product action is taken. The Standard Procedures for Bacteriological Analyses, Fisheries Services Manual requires the use of the IMViC series of tests to confirm the presence of E. coli before such action is taken. This series takes nine days for final confirmation, with the limiting factor in this series being the methyl red test. Problems do occur since the provision of the results to importers and producers take so long. Subsequently, increased costs such as storage and interest or lost sales are prevalent, not to mention costly weekend overtime for Fisheries personnel. In addition, domestic plant action is delayed for products that are awaiting confirmation from the bacteriological laboratories. Therefore, it would be of benefit to both industry and the Fisheries Service if a quicker method of confirmation could be employed, maintaining the present accuracy. An alternate method is described in J. MacFaddin (1976). The methodology described was obtained from the original work of Barry et al. (1970). This system, if found to be accurate and approved, would decrease the time required for the methyl red test to one day, thus reducing the complete routine and confirmation analyses of the shipment from nine to six days. This is broken down as follows: inoculation into Lauryl Tryptose Broth for 24 hours - 1st day; inoculation into Ec Broth for 24 hours - 2nd day; plated onto Eosin Methylene Blue agar for 24 hours - 3rd day; confirmation by inoculation of a suspect test colony into the IMViC series of biochemical tests - 4th day; incubation and interpretation of these test reactions -- Indole after 24 hours - 5th day, Methyl Red after five days for the standard rather lengthy Methyl Red test - 9th day, or after 24 hours for the Rapid Methyl Red test - 5th day, Voges Proskauer after 48 hours - 6th day, and Citrate after 24 hours and 48 hours - 6th day.

The two methods are essentially the same in that the principle, purpose, biochemistry involved, media and reagent employed and method of interpretation can be used for both. Only the amount of media, reagent, and time of incubation are different. The Rapid Methyl Red tests were completed using the Difco media presently in use in the laboratory.

PRINCIPLE AND PURPOSE

The main principle of the methyl red test, according to MacFaddin (1976, p. 133), is to determine the ability of an organism to produce and maintain stable acid end products from glucose fermentation and to overcome the buffering capacity of the system. The acid production is determined by the pH and is a qualitative measurement. (Some organisms will produce more acids than others.) As described in the Standard Procedures Manual Appendix P, (see Appendix 1 this report), the methyl red test aids in the differentiation of Escherichia coli and Escherichia freundii (Intermediates) from Aerobacter aerogenes.

BIOCHEMISTRY

The methyl red test is based on the use of a pH indicator, methyl red, to determine the hydrogen ion concentration (pH) present when an organism ferments glucose. The resulting pH is an index to the different pathways of glucose metabolism exhibited by various organisms. These different fermentation patterns are due to variations in enzymes present in the organism.

All members of the Enterobacteriaceae are glucose fermenters. Therefore, after incubation in Methyl Red (MR)/Voges Proskauer (VP) broth for 18 to 24 hours, they will initially give a positive methyl red reaction. However after further incubation, up to five days, those organisms that are methyl red positive continue to produce stable acids maintaining a high concentration of hydrogen ions (low pH) until a certain concentration is reached and then all activity ceases. The methyl red negative organisms still produce acids but these organisms continue to further metabolize the acids and revert towards neutrality, thus lowering the hydrogen ion concentration (high pH). From this, it is obvious that the validity of the methyl red test depends upon a sufficient incubation period in order to permit the difference in glucose metabolism to occur. According to MacFaddin (1976, p. 136), studies have shown that by using smaller broth volumes, the test organisms had better exposure to the atmospheric oxygen which caused the methyl red negative organisms to revert towards neutrality at a faster rate; thus the rapid methyl red test came into use.

INTERPRETATION

A methyl red positive test occurs when the culture broth is sufficiently acid to allow the methyl red reagent to remain a distinct red color (pH 4.4) at the surface of the medium. A methyl red negative test is indicated by a distinct yellow color (pH 6.0) at the surface of the medium. If a delayed reaction, characterized by an orange color, occurs the incubation should be extended. This often results in an extremely turbid broth where bacterial growth is inhibited. Therefore, more time is required for methyl red organisms to accumulate enough acid products.

COMPARISON TESTING

The rapid methyl red tests were run in conjunction with the routine testing to determine if any variance occurred. Every sample, in a period of approximately one year, (from August, 1978 to November, 1979), with a fecal coliform count greater than 4/gram was tested in this manner. It was thought that during this period nearly every routine situation would have arisen, thus proving or disproving the rapid

methyl red test's accuracy and value to the laboratory.

One suspect colony was aseptically removed from the EMB agar and inoculated into the IMViC series. This was completed using a sterile straight wire as opposed to a loop. The wire minimized the transfer of media and enabled the entire IMViC series plus the rapid MR tube to be inoculated with a single colony. Thus, the possible selection of different bacteria was eliminated. The wire containing the colony was touched to the side of the IMViC tubes just below the media. In this manner one was assured of a proper inoculation since the colonies could be seen when adhered to the side of the test tube as indicated by a slightly purple mark. Immediately after inoculation into the routine MR-VP broth tube, the same colony was inoculated into the rapid MR-VP tube. The rapid MR-VP tube was prepared just prior to use by aseptically pipetting 0.5 mL aliquots of sterile MR-VP broth into small, sterile test tubes (13 x 100 mm). Controls were run with each testing of the rapid MR-VP. This series together with the rapid MR-VP tests were incubated at 35°C. After an incubation period of 18 to 24 hours, one drop of the methyl red indicator was added to each rapid MR tube set up including the control and results interpreted immediately. The controls were closely monitored to ensure the method and reagents were operating effectively. These were later compared to the routine tests (described in the Fisheries Services Manual) which were interpreted after a further four day incubation period. In the routine tests, the small test tubes received 4 mL of the MR-VP broth and consequently five drops of methyl red indicator were added.

CONCLUSION

The results prove that the rapid methyl red test gives identical results when compared to the routine methyl red test. (The test results were easily interpreted with no orange hues present.) These comparisons were carried out on raw battered, breaded and non-breaded products; cooked battered and breaded products in addition to a variety of fresh frozen fish ranging from domestic to imports (see Appendix 2). In all cases, the correlation of results on Escherichia coli Variety I and II, E. freundii Variety I and II, Aerobacter aerogenes plus some others which were not identified by our identification key was exact. This method has been approved for inclusion in the Standard Procedures for Bacteriological Analyses. The saving in time for products under detention has amounted to three days. Such a gain of three days has allowed the importer or producer to move his products more quickly and thus save money for himself and ultimately the consumer. In addition there was a monetary saving to the Fisheries Services due to a reduction of overtime. As a bonus there was a reduction in the amount of media and reagents by 87.5 and 80.0%, respectively, and therefore, a further saving in taxpayers dollars.

The use of the rapid methyl red test makes good economic sense for all concerned without sacrificing accuracy.

ACKNOWLEDGMENTS

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BARRY, A. L., K. L. BERNSOHN, A. P. ADAMS and L. D. THRUPP. 1970. Improved 18 hour methyl red test. Appl. Microbiol. 20 (6): 866-870.

MacFADDIN, J. 1976. Biochemical test for identification of medical bacteria. Williams and Wilkins Company, Baltimore, MD. p. 133-137.

A literature search on rapid methods used to confirm E. coli was completed by the Freshwater Institute Library. Several references and new methods were uncovered. However, many of these methods involved complex procedures and/or required the purchase of new equipment. The Rapid Methyl Red Technique is based on current techniques used in the Fisheries and Oceans Bacteriological Laboratories and therefore will not require intensive training programs or new supplies.

Appendix 1. Standard procedures for bacteriological analyses, Fisheries Services Manual.

APPENDIX P

INTERPRETATION OF IMViC REACTIONS

Organism	Indole	Methyl Red	Voges-Proskauer	Citrate
Escherichia coli				
Variety I	+	+	-	-
Variety II	-	+	-	-
Escherichia freundii (Intermediates)				
Variety I	-	+	-	±
Variety II	+	+	-	+
Aerobacter aerogenes				
Variety I	-	-	+	±
Variety II	±	-	+	+

Details of Reactions:

1. Indole-Use 24 hour incubation of 1% tryptone. Run 0.5 ml of Kovac's Reagent down side of tube to form a layer on the surface of the medium.
   - Wine red colour.....positive
   - Unchanged (yellowish-green).....negative

2. Methyl Red-Use 5 day incubation in MR-VP medium. Add 2-5 drops of methyl red solution and shake.
   - Red colour.....positive
   - Yellow colour.....negative

3. Voges-Proskauer (Acetyl methyl carbinol)-Use 48 hour incubation in 3-5 ml of MR-VP medium. Add 6 drops of a-Naphthol solution and agitate the tube. The contents will become turbid. Add 3 drops of 40% KOH and agitate by snapping the tubes vigorously so that the tube contents are strongly oxygenated. The contents will probably be clear, with some persistent surface foam. Re-incubate at 35°C for not longer than 4 hours.
   - Red colour.....positive
   - Unchanged.....negative

4. Citrate-Inoculate Simmons Citrate Agar slants by needle with a single surface stroke. Read results after 24 and 48 hours incubation at 35°C.
   - Blue colour in medium.....positive
   - Green(unchanged).....negative

Appendix 2. Comparison results of routine MR to rapid MR*.

Description of Sample		*E. coli* Variety I & II	*A. aerogenes* Variety I & II	*E. freundii* Variety I & II	Other
I	RAW				
	Battered				
	Unknown Fish	4:4			
	Breaded				
	Cod	4:4			
	Shrimp	30:30	15:15		3:3
	Non-Breaded				
	Fillets - Grenadere	50:50		1:1	1:1
	Pickerel	4:4			
	Red Snapper	19:19			
	Minced - Carp	81:81			
	Mullet	48:48			2:2
	Northern Pike	50:50		5:5	2:2
	Sauger	6:6			
	Walleye	21:21			1:1
	Whitefish	71:71		5:5	
	Shrimp	12:12	18:18	3:3	13:13
	Moonfish	3:3	2:2	2:2	1:1
II	COOKED				
	Battered				
	Cod	6:6			
	Breaded				
	Unknown Fish	4:4			
III	OTHERS				
	Check Sample	4:4			
	Swabs	6:6	9:9		

* Results are shown as a ratio - routine methyl red : rapid methyl red.

TOTAL 507:507

The rapid methyl red test presented identical results when compared to the routine methyl red test in the 507 analyses completed.

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